

# TREATMENT OF HUMAN MULTIPLE MYELOMA

## BY CURCUMIN

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### Cross-Reference to Related Application

This non-provisional application claims benefit of  
10 provisional application U.S. Serial No. 60/390,926, filed June 24,  
2002, now abandoned.

### BACKGROUND OF THE INVENTION

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### Field of the Invention

The present invention relates generally to the field of cancer biology. More specifically, the present invention discloses methods of treating human multiple myeloma by curcumin.

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### Description of the Related Art

Multiple myeloma is a B cell malignancy characterized by the latent accumulation in bone marrow of secretory plasma cells with a low proliferative index and an extended life span. Multiple myeloma accounts for 1% of all cancers and >10% of all hematologic cancers. Standard treatment regimen includes a combination of vincristine, BCNU, melphalan, cyclophosphamide, Adriamycin, and prednisone or dexamethasone. Despite treatment with large doses of glucocorticoids and alkylating agents, this malignancy remains incurable. Complete remission rate is 5% and median survival is 30-36 months. In more than 90% of the patients, the disease becomes chemoresistant. Therefore, safe and efficacious agents are urgently needed for treatment of multiple myeloma.

Dysregulation of apoptotic mechanisms in plasma cells is considered a major underlying factor in the pathogenesis and subsequent chemoresistance in multiple myeloma. It is established that IL-6, produced in either an autocrine or paracrine manner, has an essential role in the malignant progression of multiple myeloma by regulating the growth and survival of tumor cells. The presence of IL-6 leads to constitutive activation of Stat 3 which in turn results

in expression of high levels of anti-apoptotic protein Bcl-x<sub>L</sub>. Bcl-2 overexpression, another important characteristic of the majority of multiple myeloma cell lines, rescues these tumor cells from glucocorticoid-induced apoptosis. Treatment of multiple myeloma 5 cells with TNF activates NF-κB, induces secretion of IL-6, induces expression of various adhesion molecules and promotes proliferation. Furthermore, multiple myeloma cells have been shown to express the ligand for the receptor that activates NF-κB (RANKL), a member of the TNF superfamily which could mediate 10 multiple myeloma-induced osteolytic bone disease.

One of the potential mechanisms by which multiple myeloma cells could develop resistance to apoptosis is through the activation of nuclear transcription factor NF-κB. Under normal conditions, NF-κB is present in the cytoplasm as an inactive 15 heterotrimer consisting of p50, p65, and IκB $\alpha$  subunits. Upon activation, IκB $\alpha$  undergoes phosphorylation and ubiquitination-dependent degradation by the 26S proteosome, thus exposing nuclear localization signals on the p50-p65 heterodimer, leading to nuclear translocation and binding to a specific consensus DNA 20 sequence (5'-GGGACTTTC-3', SEQ ID NO. 1). NF-κB binding to DNA activates gene expression that in turn results in gene transcription.

Phosphorylation of  $I\kappa B\alpha$  occurs through the activation of  $I\kappa B$  kinase (IKK). The  $I\kappa B$  kinase complex consists of three proteins IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$ /NF- $\kappa B$  essential modulator (NEMO). IKK $\alpha$  and IKK $\beta$  are the kinases that are capable of phosphorylating  $I\kappa B\alpha$ , whereas 5 IKK $\gamma$ /NEMO is a scaffold protein that is critical for IKK $\alpha$  and IKK $\beta$  activity.

Extensive research during the past few years has indicated NF- $\kappa B$  regulates the expression of various genes that play critical roles in apoptosis, tumorigenesis, and inflammation. Some 10 of the NF- $\kappa B$ -regulated genes include  $I\kappa B\alpha$ , cyclin D1, Bcl-2,  $bcl-x_L$ , COX-2, IL-6, and adhesion molecules ICAM-1, VCAM-1, and ELAM-1. Recently it was reported that NF- $\kappa B$  is constitutively active in multiple myeloma cells, leading to Bcl-2 expression that rescues 15 these cells from glucocorticoid-induced apoptosis. Since multiple myeloma cells express IL-6, various adhesion molecules,  $Bcl-x_L$ , and Bcl-2 which are all regulated by NF- $\kappa B$ , and since their suppression can lead to apoptosis, it is proposed that NF- $\kappa B$  is an important target for multiple myeloma treatment. However, the prior art is 20 deficient in identifying a pharmacologically safe and effective agent with which to block constitutive NF- $\kappa B$  in multiple myeloma. The present invention fulfills this long-standing need in the art.

## SUMMARY OF THE INVENTION

Because of the central role of nuclear transcription factor NF- $\kappa$ B in cell survival and proliferation, the possibility of using it as a target for multiple myeloma treatment was explored by using curcumin (diferuloylmethane), an agent known to have very little or no toxicity in humans. NF- $\kappa$ B was constitutively active in all human multiple myeloma cell lines examined and that curcumin, a chemopreventive agent, downregulated NF- $\kappa$ B in all cell lines as indicated by electrophoretic mobility gel shift assay and prevented nuclear retention of p65 as shown by immunocytochemistry. All multiple myeloma cell lines showed constitutively active I $\kappa$ B kinase (IKK) and I $\kappa$ B $\alpha$  phosphorylation. Curcumin suppressed constitutive I $\kappa$ B $\alpha$  phosphorylation through inhibition of I $\kappa$ B kinase activity. Curcumin also downregulated expression of NF- $\kappa$ B-regulated gene products including I $\kappa$ B $\alpha$ , Bcl-2, Bcl-x<sub>L</sub>, cyclin D1 and interleukin-6. This led to suppression of proliferation and arrest of cells at the G1/S phase of the cell cycle.

Suppression of NF- $\kappa$ B complex by IKK $\gamma$ /NF- $\kappa$ B essential modulator-binding domain peptide also suppressed proliferation of

multiple myeloma cells. Curcumin also induced apoptosis as indicated by activation of caspase-7 and caspase-9 and by PARP cleavage. Curcumin-induced downregulation of NF-κB, a factor that has been implicated in chemoresistance, also induced 5 chemosensitivity to vincristine. These results indicate that curcumin downregulates NF-κB in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis.

The present invention also assayed CD138<sup>+</sup> cells from the bone marrow of 22 multiple myeloma patients and checked for 10 activated form of NF-κB and STAT3 by immunocytochemistry. It was found that multiple myeloma cells from all the patients expressed the activated forms of NF-κB and STAT3. Constitutive activation of NF-κB was independently confirmed by electrophoretic mobility gel shift assay. In contrast to multiple myeloma patients, NF-κB and 15 STAT3 were absent in cells from healthy individuals. Suppression of the activation of NF-κB and STAT3 in multiple myeloma cells by ex vivo treatment with curcumin (diferuloylmethane) resulted in a decrease in the viability of cells. Dexamethasone partially suppressed NF-κB activation and was minimally cytotoxic to 20 myeloma cells. Overall, these results indicate that fresh cells from multiple myeloma patients express constitutively active NF-κB and

STAT3, and suppression of these transcription factors inhibits the survival of these cells.

Other and further aspects, features, and advantages of the present invention will be apparent from the following 5 description of the presently preferred embodiments of the invention. These embodiments are given for the purpose of disclosure.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1** shows curcumin inhibits constitutive nuclear NF- $\kappa$ B in multiple myeloma. **Figure 1A:** Dose response of NF- $\kappa$ B to curcumin treatment of U266 cells. Two million cells/ml were 15 treated with the indicated concentrations of curcumin for 4 h and tested for nuclear NF- $\kappa$ B by EMSA. **Figure 1B:** Effect of exposure duration on curcumin-induced NF- $\kappa$ B suppression in U266 cells. Cells were treated with curcumin (50  $\mu$ M) for the indicated times 20 and tested for nuclear NF- $\kappa$ B by EMSA. **Figure 1C:** Effect of exposure duration on curcumin-induced NF- $\kappa$ B suppression in MM.1 cells. Cells were treated as described in Fig. 1B. **Figure 1D:** Effect

of exposure duration on curcumin-induced NF- $\kappa$ B suppression in MM.1R cells. Cells were treated as described in **Figure 1B**. **Figure 1E**: Effect of exposure duration on curcumin-induced NF- $\kappa$ B suppression in RPMI 8226. Cells were treated as described in **Figure 5 1B**. **Figure 1F**: The binding of NF- $\kappa$ B to DNA is specific and consists of p50 and p65 subunits. Nuclear extracts were prepared from U266 cells ( $2 \times 10^6$ /ml), incubated for 30 min with different antibodies or unlabeled NF- $\kappa$ B oligonucleotide probe, and then assayed for NF- $\kappa$ B by EMSA.

10 **Figure 2** shows that curcumin induces redistribution of p65. U266 and RPMI 8226 cells were incubated alone or with curcumin ( $50 \mu\text{M}$ ) for 4 hours and then analyzed for the distribution of p65 by immunocytochemistry. Red stain indicates the localization of p65 and blue stain (Hoechst) indicates nucleus 15 (magnification 200X).

**Figure 3** shows that curcumin inhibits I $\kappa$ B $\alpha$  phosphorylation and I $\kappa$ B kinase. Five million U266 cells/2.5 ml were treated with curcumin ( $50 \mu\text{M}$ ) for the indicated times and cytoplasmic extracts were prepared. **Figure 3A**: Level of 20 phosphorylated I $\kappa$ B $\alpha$  determined by Western blotting. **Figure 3B**: Immunoprecipitated I $\kappa$ B kinase and kinase assay of I $\kappa$ B kinase

activity (upper panel) or Western blotting for the analysis of total IKK $\alpha$  and IKK $\beta$  proteins in cytoplasmic extracts (lower panel).

**Figure 3C:** I $\kappa$ B kinase was immunoprecipitated and kinase assay was performed in the absence or presence of the indicated

5 concentrations of curcumin (upper panel). Lower panel indicates the amount of GST-I $\kappa$ B $\alpha$  protein stained with Coomassie Blue in each well in the same dried gel.

**Figure 4** shows the effect of curcumin on NF- $\kappa$ B regulated gene products. Two million U266 cells were treated with

10 curcumin (50  $\mu$ M) for the indicated times, and cytoplasmic extracts were prepared. Sixty micrograms of cytoplasmic extracts were resolved on 10% SDS-PAGE gel, electrotransferred on a nitrocellulose membrane, and probed for the following: I $\kappa$ B $\alpha$

(Figure 4A); Bcl-2 (Figure 4B); Bcl-x $_L$  (Figure 4C) and cyclin D1

15 (Figure 4D). The same blots were stripped and reprobed with anti- $\beta$ -actin antibody to show equal protein loading (lower panel in each figure).

**Figure 4E:** Curcumin downregulates IL-6 production.

U266, MM.1 or RPMI 8226 cells ( $1 \times 10^7$ ) were treated with curcumin (10 mM) in 5 ml of medium for the indicated times.

20 Supernatants were harvested and concentrated approximately 20 X, and IL-6 was quantitated by an IL-6 ELISA kit. Values shown were

normalized to 1 ml of un-concentrated supernatants.

Figure 5 shows the curcumin inhibits the growth of human multiple myeloma cells. U266 (Figure 5A); RPMI 8226 (Figure 5B); MM.1 (Figure 5C) or MM.1R cells (Figure 4D) (5000 cells/0.1 ml) were incubated at 37°C with curcumin (1 mM or 10 mM) for the indicated times and viable cells were counted using standard trypan blue dye exclusion test. The results are shown as the mean ( $\pm$  s. d.) cell count from triplicate cultures.

Figure 6 shows that curcumin inhibits the growth of human multiple myeloma cells and induces apoptosis. Figure 6A: U266 cells (5000 cells/0.1 ml) were incubated with different concentrations of curcumin for 24 hours, and cell proliferation assay was performed as described. Results are shown as mean ( $\pm$  s.d.) of percent [ $^3$ H]-thymidine incorporation of triplicate cultures compared to untreated control. Figure 6B: U266 cells (5000 cells/0.1 ml) were incubated with different concentrations of curcumin for 24 hours, and cell viability was determined by MTT method. The results are shown as the mean ( $\pm$  s.d.) percent viability from triplicate cultures.

Figure 6C-E shows that U266 cells ( $2 \times 10^6$  cells/ml) were incubated in the absence or presence of curcumin (50  $\mu$ M) for

indicated times. The cells were washed and total proteins were extracted by lysing the cells. Sixty microgram of extracts were resolved on 10% SDS-PAGE gel, electrotransferred to a nitrocellulose membrane, and probed with anti-procaspase-9 (Figure 6C), anti- 5 procaspase-7 (Figure 6D), anti-PARP (Figure 6E, upper panel) and anti-cleaved PARP (Figure 6E, lower panel).

Figure 7 shows that curcumin arrests cells at the G1/S phase of the cell cycle. U266 cells ( $2 \times 10^6$  cells/ml) were incubated in the absence or presence of curcumin (10  $\mu$ M) for the 10 indicated times. The cells were then washed, fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry.

Figure 8 shows that the NEMO-binding domain (NBD) peptide inhibits constitutive NF- $\kappa$ B and induces cytotoxicity in multiple myeloma cells. Figure 8A: U266 cells ( $2 \times 10^6$  cells/ml) 15 were treated with indicated concentrations of NEMO-control or NBD-peptide (100  $\mu$ M) for the indicated times. Nuclear extracts were then checked for the presence of NF- $\kappa$ B DNA-binding activity by EMSA. Figure 8B: Untreated or NBD-peptide-treated (100  $\mu$ M; 12 h) U266 cells were cytospun, and p65 immunocytochemistry was 20 performed as described. Red stain indicates the localization of p65 and blue stain indicates nucleus (magnification 200X). Figure 8C:

U266 cells ( $2 \times 10^6$  cells/ml) were treated with indicated concentrations of NEMO-control or NBD-peptide (100  $\mu$ M) for the indicated time periods, and cell viability was monitored by the trypan blue dye exclusion method. Percent cell killing was 5 determined as: (number of trypan blue stained cells/total cells) x 100.

Figure 9 shows that curcumin potentiates the cytotoxic effect of vincristine in multiple myeloma cells. U266 cells (10000 cells/0.1 ml) were incubated without or with vincristine (50  $\mu$ M) in 10 the absence or presence of curcumin (10  $\mu$ M) for 24 h, and then cell viability was determined by the MTT method.

Figure 10 shows immunocytochemical localization of NF- $\kappa$ B in human multiple myeloma cell lines (Figure 10A), peripheral blood mononuclear cells (PBMC) from healthy subjects, and bone 15 marrow CD138 $^+$  multiple myeloma cells from a patient (Figure 10B). PBMC were collected from the blood of a healthy subject by Ficoll-Paque density gradient centrifugation. CD138 $^+$  cells were enriched from bone marrow aspirates of multiple myeloma patient (patient #1), enriched by magnetic bead separation method, and 20 immunostained for NF- $\kappa$ B (p65). Red stain indicates specific

staining for NF- $\kappa$ B, whereas blue stain indicates the relative position of the nuclei in the corresponding view.

**Figure 11** shows nuclear localization of NF- $\kappa$ B in bone marrow CD138 $^{+}$  cells from multiple myeloma patients. Enriched 5 CD138 $^{+}$  cells from bone marrow aspirates of different multiple myeloma patients were immunostained for NF- $\kappa$ B (p65) (Figure 11A). Red stain indicates specific staining for NF- $\kappa$ B, whereas blue stain indicates a relative position of the nuclei in the corresponding view. Figure 11B shows enriched CD138 $^{+}$  cells ( $2 \times 10^6$  cells) from 10 bone marrow aspirates of a multiple myeloma patient (patient #4) were tested for NF- $\kappa$ B activity in the nuclei by electrophoretic mobility shift assay. Untreated or TNF-treated KBM-5 (TNF-1nM, 30 min) was used as negative and positive controls respectively.

**Figure 12** shows nuclear localization of STAT3 in 15 multiple myeloma cell lines (Figure 12A), PBMCs and bone marrow CD138 $^{+}$  multiple myeloma cells from patients (Figures 12B-C). Enriched CD138 $^{+}$  cells from bone marrow aspirates of different multiple myeloma patients were immunostained for STAT3 as described below. Red stain indicates specific staining for STAT3, 20 whereas blue stain indicates the relative position of the nuclei in the corresponding view.

Figure 13 shows curcumin prevents nuclear localization of NF- $\kappa$ B and STAT3 in bone marrow CD138 $^{+}$  multiple myeloma cells. Enriched CD138 $^{+}$  cells ( $1 \times 10^5$  cells/0.1 ml) from bone marrow aspirates of multiple myeloma patients #5, #9, or #10 were cultured 5 in the absence or presence of curcumin (50  $\mu$ M) for 1 h for STAT3 analysis or for 2h for NF- $\kappa$ B analysis. The cells were fixed on slides by cytospin centrifugation and immuno-stained for NF- $\kappa$ B or STAT3. Red stain indicates specific staining for NF- $\kappa$ B or STAT3, whereas blue stain indicates the relative position of the nuclei in the 10 corresponding view.

Figure 14 shows curcumin inhibits the growth/viability of human multiple myeloma cell line U266 and bone marrow CD138 $^{+}$  multiple myeloma cells. Cell line U266 (Figure 14A) or enriched CD138 $^{+}$  cells ( $2 \times 10^4$  cells/0.1 ml) from bone marrow 15 aspirates of multiple myeloma patients #7, #9, or #10 (Figures 14B-D) were cultured in the absence or presence of the indicated concentrations of curcumin for 24h and cell viability was measured by MTT assay (Figures 14A, B) or standard Trypan blue dye exclusion method (Figures 14C, D).

Figure 15 shows the effect of curcumin and dexamethasone on nuclear localization of NF- $\kappa$ B and STAT3 in bone

marrow CD138<sup>+</sup> multiple myeloma cells. Enriched CD138<sup>+</sup> cells (1x10<sup>5</sup> cells/0.1 ml) from bone marrow aspirates of multiple myeloma patient #20 were cultured in the absence or presence of curcumin or dexamethasone (50 µM each) for 2 h, and cells were 5 fixed on slides by cytospin centrifugation and immunostained for NF-κB or STAT3. Red stain indicates the specific staining for NF-κB or STAT3 as indicated, whereas blue stain indicates the relative position of the nuclei in the corresponding view.

Figure 16 shows the effect of curcumin and 10 dexamethasone on growth/viability of bone marrow CD138<sup>+</sup> multiple myeloma cells. Enriched CD138<sup>+</sup> cells (2x10<sup>5</sup> cells/0.1 ml) from bone marrow aspirates of multiple myeloma patient #20 were cultured in the absence or presence of indicated concentrations of curcumin (Figure 16A) or dexamethasone (Figure 16B) for 24 h, and 15 cell viability was measured by MTT assay.

## DETAILED DESCRIPTION OF THE INVENTION

Curcumin has been shown to suppress NF- $\kappa$ B activation induced by various inflammatory stimuli and inhibit activation of 5 I $\kappa$ B kinase activity needed for NF- $\kappa$ B activation. Curcumin also downregulates expression of various NF- $\kappa$ B-regulated genes including bcl-2, COX2, MMP-9, TNF, cyclin D1 and adhesion molecules. Moreover, curcumin has been reported to induce apoptosis in a wide variety of cells through sequential activation of 10 caspase-8, BID cleavage, cytochrome C release, caspase-9, and caspase-3. Numerous studies in animals have demonstrated that curcumin has potent chemopreventive activity against a wide variety of different tumors (Rao et al., 1995; Kawamori et al., 1999), and administration of curcumin in humans even at 8 g per day has been 15 shown to be safe in phase I clinical trials (Cheng et al., 1998).

Results presented herein indicate that NF- $\kappa$ B is constitutively active in all human multiple myeloma cell lines examined. Curcumin downregulated the nuclear pool, or active form of NF- $\kappa$ B and suppressed constitutive I $\kappa$ B $\alpha$  phosphorylation, 20 IKK kinase activity, and expression of NF- $\kappa$ B-regulated gene products I $\kappa$ B $\alpha$ , Bcl-2, Bcl-x<sub>L</sub>, cyclin D1, and interleukin-6. This led to

suppression of proliferation, arrest of cells at the G1/S phase boundary of the cell cycle, and induction of apoptosis as indicated by the activation of caspase-7 and caspase-9 and PARP cleavage. Curcumin also induced chemosensitivity to vincristine.

5 All four multiple myeloma cell lines (U266, RPMI8226, MM.1 and MM.1R) used herein expressed constitutively active NF- $\kappa$ B. These results are in agreement with two recent reports that showed constitutive NF- $\kappa$ B in U266 and RPMI-8226 cells by electrophoretic mobility gel shift assay. MM.1 and MM.1R, a 10 dexamethasone-resistant cell line, also express constitutive NF- $\kappa$ B. These results differ from those of Hideshima et al., who showed lack of constitutively active NF- $\kappa$ B in MM.1S cells which are same as MM.1 cells. Because constitutive activation of NF- $\kappa$ B leads to nuclear translocation of p65, the presence of nuclear p65 in all the 15 cell lines examined by immunocytochemistry was confirmed. These results further indicate that multiple myeloma cells exhibit constitutively active I $\kappa$ B kinase which is the kinase required for NF- $\kappa$ B activation. This is the first report to show an elevated I $\kappa$ B kinase activity in multiple myeloma cells.

20 Suppression of constitutive NF- $\kappa$ B activation by curcumin in all four multiple myeloma cell lines examined herein is in

agreement with previous reports that showed curcumin is a potent inhibitor of NF- $\kappa$ B activation. Curcumin inhibits NF- $\kappa$ B activation by blocking constitutively active I $\kappa$ B kinase present in multiple myeloma cells. Because curcumin inhibited I $\kappa$ B kinase activity both 5 inside the cells and *in vitro*, it is suggested that curcumin may be a direct inhibitor of I $\kappa$ B kinase. Since recombinant I $\kappa$ B kinase enzyme was not employed, one can not completely rule out the possibility of indirect inhibition of I $\kappa$ B kinase by curcumin. In any case, curcumin appears to suppress I $\kappa$ B kinase activation which leads to inhibition 10 of I $\kappa$ B $\alpha$  phosphorylation and thus abrogation of I $\kappa$ B $\alpha$  degradation. These results are in agreement with previous reports which showed inhibition of I $\kappa$ B kinase by curcumin in colon cancer cells and macrophages. A recent report showed that PS-1145, a rationally designed I $\kappa$ B kinase inhibitor, blocked TNF-induced NF- $\kappa$ B activation 15 in MM.1 cells. The concentration of curcumin required to block I $\kappa$ B kinase activity in the cells was comparable to that reported for PS-1145.

Suppression of cell proliferation by curcumin in multiple myeloma cells is in agreement with previous reports that showed 20 curcumin-induced suppression of NF- $\kappa$ B leads to inhibition of cellular proliferation of cutaneous T cell lymphoma and acute

myelogenous leukemia. The results on the antiproliferative effects of curcumin are in agreement with those of Hideshima et al. who showed PS-1145, an IKK blocker, inhibits cell proliferation. These workers reported that 50  $\mu$ M PS-1145 inhibits proliferation of 5 multiple myeloma cell lines MM.1S, RPMI-8226 and U266 by less than 50%. In contrast, almost complete inhibition of proliferation of all these cell lines was found with as little as 10  $\mu$ M curcumin.

Several potential mechanisms could explain why NF- $\kappa$ B downregulation by curcumin leads to suppression of proliferation of 10 multiple myeloma cells. One of the potential mechanisms involves suppression of IL-6 production as shown herein. Numerous studies indicate that IL-6 is a potent growth factor for multiple myeloma cells. Whether IL-6 is a paracrine or an autocrine growth factor for multiple myeloma cells is highly controversial. In these studies it is 15 unlikely, however, that curcumin suppressed the growth of multiple myeloma cells through suppression of IL-6 production because three out of the four cell lines examined produced no detectable IL-6. It is also unlikely that curcumin inhibits cell growth through downregulation of the constitutively active Stat3 signaling because 20 proliferation of cells which do not express constitutively active Stat3 (e.g; RPMI 8226) are also inhibited by curcumin. In this study,

curcumin downregulated bcl-2 and bcl-x<sub>L</sub> expression, the proteins that have been implicated in the cell survival of multiple myeloma cells. Thus it is possible that downregulation of bcl-2 and bcl-x<sub>L</sub> by curcumin could lead to suppression of multiple myeloma cell 5 proliferation.

It was also found that multiple myeloma cells overexpress cyclin D1, another NF-κB-regulated gene, and that this expression is downregulated by curcumin. Overexpression of cyclin D1 has been noted in a wide variety of tumors, but its role in 10 multiple myeloma cells has not been reported. Given that cyclin D1 is needed for cells to advance from the G1 to S phase of the cell cycle, induction of G1/S arrest and thus suppression of multiple myeloma cell proliferation by curcumin may very well resulted from downregulation of cyclin D1.

15 Suppression of NF-κB by curcumin also led to apoptosis of multiple myeloma cells as indicated by activation of caspases and cleavage of PARP. These results are in agreement with reports indicating that NF-κB mediates antiapoptotic effects. Downregulation of NF-κB also sensitized multiple myeloma cells to 20 vincristine. Even the MM.1R cells, which have been shown to be resistant to dexamethasone, were sensitive to curcumin.

Multiple myeloma is an incurable aggressive B cell malignancy, and more than 90% of multiple myeloma patients become chemoresistant. Several agents have been tested in the search for more effective treatment of multiple myeloma. These 5 include PS341, a proteosome inhibitor, and thalidomide, an inhibitor of TNF production. Nonspecific drug-toxicity is one of the major problems in drug development. However, numerous studies have shown that curcumin is pharmacologically safe. It was recently demonstrated in phase 1 clinical trials that humans can tolerate up 10 to 8 grams of curcumin per day when taken orally (Cheng et al., 1998). Furthermore, curcumin has been shown to downregulate the expression of ICAM-1, VCAM-1 and ELAM-1, all NF- $\kappa$ B-regulated gene products that have been implicated in activation of stromal cells by multiple myeloma cells. TNF, another cytokine known to play a 15 pathological role in multiple myeloma, has also been shown to be downregulated by curcumin. The results presented herein clearly demonstrate that curcumin can suppress NF- $\kappa$ B, IKK, bcl-2, bcl-x<sub>L</sub>, cyclin D1 and cell proliferation in multiple myeloma cells. These results provide enough rationale for considering curcumin worthy 20 of clinical trial in patients with multiple myeloma.

As used herein, “multiple myeloma cells” refer to

multiple myeloma cell lines or CD138<sup>+</sup> plasma cells isolated from multiple myeloma patients.

In the present invention, there are provided methods of inhibiting proliferation of multiple myeloma cells, inducing 5 apoptosis in multiple myeloma cells and increasing the cytotoxic effects of chemotherapeutic agent against multiple myeloma cells by treatment with curcumin. In general, the chemotherapeutic agent can be vincristine, BCNU, melphalan, cyclophosphamide, Adriamycin, prednisone or dexamethasone.

10 The present invention is also directed to methods of treating multiple myeloma in an individual and increasing the cytotoxic effects of chemotherapeutic agent against multiple myeloma cells in an individual by treatment with curcumin. In general, the chemotherapeutic agents are those listed above.

15 It is specifically contemplated that methods of the present invention utilize pharmaceutical compositions comprising curcumin, e.g. a pharmaceutical composition comprising curcumin and a pharmaceutically acceptable carrier that is well known and routinely used in the art. In view of the published clinical trials 20 (Cheng et al., 1998) and other studies involving the use of curcumin, a person having ordinary skill in this art would readily be

able to determine, without undue experimentation, the appropriate dosages and routes of administration of curcumin in the methods of the present invention. When used *in vivo* for therapy, curcumin is administered to the patient or an animal in therapeutically effective amounts, i.e., amounts that inhibit proliferation of multiple myeloma cells, induce apoptosis in multiple myeloma cells or increase cytotoxic effects of chemotherapeutic agent against multiple myeloma cells, e.g., administered in a dose of from about 0.01 mg/kg of the individual's body weight to about 500 mg/kg of the individual's body weight.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. The present examples, along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. Changes therein and other uses which are encompassed within the spirit of

the invention as defined by the scope of the claims will occur to those skilled in the art.

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**EXAMPLE 1**

**Cells And Reagents**

Human multiple myeloma cell lines U266, RPMI 8226, and MM.1 were obtained from the American Type Culture Collection (Rockville, MD). Cell lines U266 (ATCC#TIB-196) and RPMI 8226 (ATCC#CCL-155) are plasmacytomas of B cell origin. U266 is known to produce monoclonal antibodies and IL-6. RPMI 8226 produces only immunoglobulin light chains and there is no evidence for heavy chain or IL-6 production. Doxorubicin (Dox-6)- and melphalan (LR-5)-resistant clones of RPMI 8226 were provided by Dr. Willium Dalton (H. Lee Moffitt Cancer Center and Research Institute, Tampa, FL.)

The MM.1 (also called MM.1S) cell line, which is established from the peripheral blood cells of a patient with IgA myeloma, secretes lambda light chain, is negative for the presence of EBV genome, and expresses leukocyte antigen DR, PCA-1, T9 and T10 antigens. MM.1R is a dexamethasone-resistant variant of MM.1

cells. These two cell lines was provided by Dr. Steve T. Rosen of Northwestern University Medical School (Chicago, IL).

Rabbit polyclonal antibodies to IkBa, p50, p65, cyclin D1, Bcl-2, Bcl-x<sub>L</sub>, and PARP and STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against cleaved-PARP, phospho-IkBa, procaspase-7, procaspase-9 and the polynucleotide kinase kit were purchased from New England Biolabs, Inc. (Beverly, MA). Anti-IKK $\alpha$  and anti-IKK $\beta$  antibody were kindly provided by Imgenex (San Diego, CA). Goat anti-rabbit-HRP conjugate was purchased from Bio-Rad Laboratories (Hercules, CA), goat anti-mouse-HRP was purchased from Transduction Laboratories (Lexington, KY) and goat anti-rabbit-Alexa 594 was purchased from Molecular Probes (Eugene, OR). Anti-CD138 microbeads and PE-conjugated anti-CD138 were purchased from Miltenyi Biotech (Auburn, CA.)

Cell-permeable NEMO (NF- $\kappa$ B essential modifier; also called IKK $\gamma$ )-binding domain peptide (NBD), NH<sub>2</sub>-DRQIKIWFQNRRMKWKKTALDWSWLQTE-CONH<sub>2</sub>, (SEQ ID NO. 2) and control peptide NEMO-C, NH<sub>2</sub>-DRQIKIWFQNRRMKWKK-CONH<sub>2</sub> (SEQ ID NO. 3) were obtained from Imgenex (San Diego, CA).

Curcumin was purchased from LKT Laboratories, Inc. (St.

Paul, MN.) and was prepared as a 20 mM solution in dimethyl sulfoxide and then further diluted in cell culture medium. Vincristine, Hoechst 33342 and MTT were purchased from Sigma-Aldrich Chemicals (St. Louis, MO). RPMI-1640, fetal bovine serum (FBS), 0.4% trypan blue vital stain and 100X antibiotic-antimycotic mixture were obtained from Life Technologies Inc. (Grand Island, NY). Protein A/G-Sepharose beads were obtained from Pierce (Rockford, IL). g-P<sup>32</sup>-ATP was from ICN Pharmaceuticals (Costa Mesa, CA). Human IL-6 kit was purchased from BioSource International (Camarillo, CA). Ultrafree 4 centrifugal filters were purchased from Millipore Corporation (Bedford, MA).

All the human multiple myeloma cell lines were cultured in RPMI 1640 medium containing 1 X antibiotic-antimycotic. U266, MM.1, and MM.1R were cultured in 10% FBS, whereas cell line RPMI 8226 was grown in 20% FBS. Occasionally, cells were examined by Hoechst staining and by custom PCR for mycoplasma contamination.

## EXAMPLE 2

### 20 Preparation of Nuclear Extracts For NF-κB

Nuclear extracts were prepared according to Bharti et al.

(2003). Briefly,  $2 \times 10^6$  cells were washed with cold PBS and suspended in 0.4 ml of hypotonic lysis buffer containing protease inhibitors for 30 min. The cells were then lysed with 12.5  $\mu$ l of 10% Nonidet P-40. The homogenate was centrifuged, and supernatant 5 containing the cytoplasmic extracts was stored frozen at -80°C. The nuclear pellet was resuspended in 25- $\mu$ l ice-cold nuclear extraction buffer. After 30 min of intermittent mixing, the extract was centrifuged, and supernatants containing nuclear extracts were collected. Protein content was measured by the Bradford method.

10 If the supernatants were not used immediately, they were stored at -80 °C.

### EXAMPLE 3

#### 15 Electrophoretic Mobility Shift Assay For NF- $\kappa$ B

NF- $\kappa$ B activation was analyzed by electrophoretic mobility gel shift assay (EMSA) as described previously (Chaturvedi et al., 1994). In brief, 8- $\mu$ g nuclear extracts prepared from curcumin-treated or untreated cells were incubated with  $^{32}$ P end-20 labeled double-stranded 45-mer of NF- $\kappa$ B oligonucleotide from human immunodeficiency virus-1 long terminal repeat (5'-

TTGTTACAAGGGACTTCCGCT GGGGACTTCCAG GGAGGCGTGG- 3', SEQ ID NO. 4) for 15 min at 37 °C, and the DNA-protein complex was resolved in a 6.6 % native polyacrylamide gel. Radioactive bands from the dried gels were visualized and quantitated by 5 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using ImageQuant software.

#### EXAMPLE 4

10

#### Immunocytochemistry For NF-κB P65 And STAT3

Untreated or treated multiple myeloma cells were plated on a poly-L-lysine-coated glass slide by centrifugation using a Cytospin 4 (Thermoshendon, Pittsburg, PA), air-dried for 1 h at 15 room temperature, and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated either with rabbit polyclonal anti-human NF-κB p65 antibody (SC-109; dilution, 1:100) or with anti-human STAT3 antibody (SC-482; dilution, 1:100). After overnight 20 incubation, the slides were washed and then incubated with goat anti-rabbit IgG-Alexa 594 (A-11037; dilution, 1:100) for 1 h and

counter-stained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma Co.) and analyzed under an epifluorescence microscope (Labophot-2, Nikon, Tokyo, Japan). Pictures were captured using Photometrics 5 Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging Corp., Downingtown PA). Cells with nuclear staining of NF-κB p65 or STAT3 were counted separately. One hundred cells were counted for each specimen, and the sample was graded on the basis of a 4-point scale: -, no nuclear 10 positive cells (0%); +, low number of nuclear positive cells (< 10%); ++, moderate number of nuclear positive cells (10-50%); +++, high number of nuclear positive cells (> 50%).

15

#### EXAMPLE 5

#### Western Blot

Thirty to fifty micrograms of cytoplasmic protein extracts prepared as described (Chaturvedi et al., 2000) were 20 resolved on 10% SDS-PAGE gel. After electrophoresis, the proteins were electrotransferred to a nitrocellulose membrane, blocked with

5% nonfat milk, and probed with antibodies against either I $\kappa$ B<sub>a</sub>, phospho-I $\kappa$ B<sub>a</sub>, Bcl-2, Bcl-x<sub>L</sub>, or cyclin D1 (1:3000) for 1 h. Thereafter, the blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally detected by 5 chemiluminescence (ECL, Amersham Pharmacia Biotech., Arlington Heights, IL).

For detection of cleavage products of PARP, whole cell extracts were prepared by lysing curcumin-treated cells in lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 10 0.1% Triton -X100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 0.4 mM PMSF, and 4 mM NaVO<sub>4</sub>). Lysates were then spun at 14000 rpm for 10 min to remove insoluble material, resolved on 7.5% gel and probed with PARP antibodies. PARP was cleaved from the 116-kDa intact protein into 85-kDa and 40-kDa peptide products. To 15 detect cleavage products of procaspase 7 and procaspase 9, whole cell extracts were resolved on 10% gel and probed with appropriate antibodies.

#### EXAMPLE 6

20

#### I $\kappa$ B Kinase Assay

The I $\kappa$ B kinase assay was performed with a modified method as described earlier (Manna et al., 2000). Briefly, 200  $\mu$ g cytoplasmic extracts were immunoprecipitated with 1  $\mu$ g of anti-IKK $\alpha$  and IKK $\beta$  antibodies each, and the immune complexes so formed were precipitated with 0.01 ml of protein A/G-Sepharose beads for 2 hour. The beads were washed first with lysis buffer and then with kinase assay buffer (50 mM HEPES pH 7.4, 20 mM MgCl<sub>2</sub>, and 2 mM DTT). The immune complex was then assayed for kinase activity using kinase assay buffer containing 20 mCi [g-P<sup>32</sup>]ATP, 10  $\mu$ M unlabeled ATP, and 2  $\mu$ g/sample glutathione S-transferase-I $\kappa$ B<sub>1-54</sub>. After incubation at 30°C for 30 min, the reaction was stopped by boiling the solution in 6x SDS sample buffer. Then the reaction mixture was resolved on 12% SDS-PAGE. Radioactive bands of the dried gel were visualized and quantitated by PhosphorImager.

To determine the total amount of IKK complex in each sample, 60 mg of cytoplasmic protein was resolved on a 7.5% acrylamide gel and then electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk protein for 1 h and then incubated with either anti-IKK $\alpha$  or anti-IKK $\beta$  antibodies for 1 h. The membrane was then washed and treated with HRP-conjugated secondary anti-mouse IgG antibody and finally

detected by chemiluminescence (Amersham Pharmacia Biotech, Arlington Heights, IL).

#### **EXAMPLE 7**

5

##### **Proliferation Assay**

The antiproliferative effects of curcumin against different multiple myeloma cell lines were determined by the MTT dye uptake method as described earlier (Manna et al., 1998).

10 Briefly, cells (5000/well) were incubated in triplicate in a 96-well plate in the presence or absence of indicated test samples in a final volume of 0.1 ml for 24 h at 37°C. Thereafter, 0.025 ml of MTT solution (5 mg/ml in PBS) was added to each well. After a 2 h incubation at 37 °C, 0.1 ml of extraction buffer (20% SDS, 50% 15 dimethylformamide) was added. Incubation was continued for overnight at 37 °C, and then the OD at 590 nm was measured using a 96-well multiscanner autoreader (Dynatech MR 5000), with extraction buffer as blank. Percent cell viability = (OD of the experiment samples/ OD of the control) X 100.

20 The antiproliferative effects of curcumin were also monitored by the thymidine incorporation method. Five thousand

cells in 100 ml medium were cultured in triplicate in 96-well plates in the presence or absence of curcumin for 24 h. Six hours before the completion of experiment, the cells were pulsed with 0.5 mCi <sup>3</sup>H-thymidine, and the uptake of <sup>3</sup>H-thymidine was monitored using 5 a Matrix-9600 b-counter (Packard Instruments, Downers Grove, IL).

#### EXAMPLE 8

##### Flow Cytometric Analysis

10 To determine the effect of curcumin on cell cycle, multiple myeloma cells were treated for different times, washed, and fixed with 70% ethanol. After incubation for overnight at -20°C, cells were washed with PBS prior to staining with propidium iodide (PI), and then suspended in staining buffer (PI, 10 mg/ml; 15 Tween-20, 0.5%; RNase, 0.1% in PBS). The cells were analyzed using a FACS Vantage flow cytometer that uses CellQuest acquisition and analysis programs (Becton Dickinson, San Jose, CA).

20

### **EXAMPLE 9**

#### **Determination of IL-6 Protein**

Supernatants were collected from untreated or  
5 curcumin-treated multiple myeloma cell cultures and concentrated  
approximately 20-fold using Ultrafree 4 centrifugal filters with  
Biomax-10K NMWL Membrane (Millipore). One hundred microliter  
aliquots were removed, and IL-6 contents were determined by an  
ELISA kit (Biosource International).

10

### **EXAMPLE 10**

#### **Curcumin Suppresses Constitutive NF- $\kappa$ B Expressed By Multiple 15 Myeloma Cells**

The NF- $\kappa$ B status in four different multiple myeloma cell  
lines was first investigated by electrophoretic mobility gel shift  
assay (EMSA). The results shown in Fig. 1 indicate that all four cell  
lines expressed constitutively active NF- $\kappa$ B, resolved as an upper and  
20 a lower bands. The effect of curcumin on constitutively active NF- $\kappa$ B was then investigated by examining the dose of curcumin

required for complete suppression of NF- $\kappa$ B. U266 cells were treated with different concentrations of curcumin for 4 h and then examined for NF- $\kappa$ B by EMSA. Densitometric analysis of the retarded radiolabeled probe showed a decrease in NF- $\kappa$ B DNA binding activity. These results showed that 50  $\mu$ M curcumin was sufficient to fully suppress constitutive NF- $\kappa$ B activation in U266 cells (Fig. 1A).

The minimum duration of exposure to curcumin required for suppression of NF- $\kappa$ B was then examined. Multiple myeloma cells were incubated with 50  $\mu$ M curcumin for different times, then nuclear extracts were prepared and examined for NF- $\kappa$ B by EMSA. The results showed that curcumin downregulated constitutive NF- $\kappa$ B in all four cell lines but with different kinetics. Complete downregulation of NF- $\kappa$ B occurred at 4 h in U266 (Fig. 1B), MM.1 (Fig. 1C) and MM.1R (Fig. 1D) cells, whereas it took 8 h to downregulate NF- $\kappa$ B in RPMI8226 cells (Fig. 1E). Curcumin downregulated only the upper band and not lower band of NF- $\kappa$ B in most cases. In the case of RPMI 8226 cells, both bands were downregulated.

Since NF- $\kappa$ B is a family of proteins, and various combinations of Rel/NF- $\kappa$ B protein can constitute an active NF- $\kappa$ B

heterodimer that binds to a specific DNA sequence. To show that the retarded band visualized by EMSA in multiple myeloma cells was indeed NF- $\kappa$ B, nuclear extracts from the multiple myeloma cells were incubated with antibody to either the p50 (NF- $\kappa$ B1) or the p65 (RelA) subunit of NF- $\kappa$ B. Both shifted the band to a higher molecular mass (Fig. 1F), thus suggesting that the major NF- $\kappa$ B band in the multiple myeloma cells consisted of p50 and p65 subunits. A nonspecific minor band which was not supershifted by the antibody was observed in some multiple myeloma cell lines. Neither 10 preimmune serum nor the irrelevant antibody such as anti-cyclin D1 had any effect. Excess unlabeled NF- $\kappa$ B (100-fold), but not the mutated oligonucleotides, caused complete disappearance of the band.

When NF- $\kappa$ B is activated, the p65 subunit of the NF- $\kappa$ B 15 containing transactivation domain is translocated to the nucleus. In the inactive state, the p65 subunit of NF- $\kappa$ B is retained in the cytoplasm. Immunocytochemistry was then used to confirm that curcumin suppresses nuclear retention of p65. Curcumin-treated and untreated cells were cytospon on a glass slide, immunostained 20 with anti-p65 antibody, and then visualized by the Alexa-594 conjugated second antibody as described above. The results in Fig.

2 clearly demonstrate that curcumin prevented the translocation of the p65 subunit of NF- $\kappa$ B to the nucleus in all four multiple myeloma cell lines. These cytological findings were consistent with the NF- $\kappa$ B inhibition observed by EMSA.

5

#### EXAMPLE 11

##### Curcumin Inhibits I $\kappa$ B $\alpha$ Phosphorylation And I $\kappa$ B Kinase Activity

The degradation of I $\kappa$ B $\alpha$  and subsequent release of NF- $\kappa$ B 10 (p65:p50) requires prior phosphorylation at ser 32 and ser 36 residues. Therefore, in order to investigate whether the inhibitory effect of curcumin is mediated through alteration of I $\kappa$ B $\alpha$  phosphorylation, U266 cells were treated with curcumin and their protein extracts were checked for phospho-I $\kappa$ B $\alpha$  expression. Results 15 in Figure 3A show that untreated U266 cells constitutively expressed ser 32-phosphorylated I $\kappa$ B $\alpha$ . Upon curcumin treatment, the phosphorylated I $\kappa$ B $\alpha$  content decreased rapidly.

Phosphorylation of I $\kappa$ B $\alpha$  is mediated through I $\kappa$ B kinase.

*In vitro* kinase assay using immunoprecipitated I $\kappa$ B kinase from 20 untreated U266 cells and GST-I $\kappa$ B $\alpha$  as substrate showed constitutive

I $\kappa$ B kinase activity, whereas under similar conditions I $\kappa$ B kinase immunoprecipitated from curcumin-treated cells showed a decreased in kinase activity that corresponded to the duration of curcumin treatment (Figure 3B; upper panel). However, 5 immunoblotting analysis of cell extracts from untreated and curcumin-treated cells showed no significant change in the protein levels of I $\kappa$ B kinase subunits IKK $\alpha$  and IKK $\beta$  (Figure 3B; middle and lower panel).

I $\kappa$ B kinase has been shown to be regulated by several 10 upstream kinases. To determine if curcumin acted as a direct inhibitor of I $\kappa$ B kinase activity, I $\kappa$ B kinase was immunoprecipitated from untreated U266 cells and then treated with different concentrations of curcumin for 30 min. After the treatment, the samples were examined for I $\kappa$ B kinase activity using GST-I $\kappa$ B $\alpha$  as a 15 substrate. Results in Figure 3C (upper panel) showed that curcumin inhibited I $\kappa$ B kinase activity directly in a dose-dependent manner. These results suggest that curcumin is a direct inhibitor of I $\kappa$ B kinase. Since purified I $\kappa$ B kinase was not used the possibility that curcumin suppressed an upstream kinase required for I $\kappa$ B kinase 20 activation can not be completely ruled out.

## EXAMPLE 12

### 5 Curcumin Downregulates The Expression Of NF-κB-Regulated Gene Products

Because  $\text{I}\kappa\text{B}\alpha$ , Bcl-2, Bcl-x<sub>L</sub>, and cyclin D1 have all been shown to be regulated by NF-κB, the effect of curcumin on the expression of these gene products was examined by 10 immunoblotting. As depicted in Fig. 4, all four gene products were expressed in U266 cells. Treating cells with curcumin downregulated the pools of  $\text{I}\kappa\text{B}\alpha$  (Figure 4A), Bcl-2 (Figure 4B), Bcl-x<sub>L</sub> (Figure 4C) and cyclinD1 (Figure 4E) proteins in a time-dependent manner, although the kinetics of suppression were different. Cyclin 15 D1 showed the most abrupt and complete depletion within 4 hours of curcumin treatment. Bcl-2 also showed a complete decline but it achieved the lowest level by 8 hours. On the other hand  $\text{I}\kappa\text{B}\alpha$  and Bcl-xL showed only a partial decline.

Interleukin-6 is another NF-κB-regulated gene and has 20 been shown to serve as a growth factor for multiple myeloma cells. U-266 cells produced a significant amount of IL-6 protein in a time-dependent manner whereas neither MM.1 nor RPMI 8226 produced

any detectable amount of IL-6 as measured by ELISA (Fig. 4E). As shown in Fig. 4E, curcumin treatment inhibited the production of IL-6 by U266 cells.

5

### EXAMPLE 13

#### Curcumin Suppresses Proliferation Of Multiple Myeloma Cells

Because NF- $\kappa$ B has been implicated in cell survival and proliferation, the effect of curcumin on the proliferation of multiple myeloma cell lines was examined. U266, RPMI 8226, MM.1, and MM.1R cells were cultured in the presence of different concentrations of curcumin, and the number of viable cells was examined by trypan blue dye-exclusion method.

Results in Figure 5 show that curcumin at a concentration as low as 1  $\mu$ M inhibited the growth of U266 (panel A), RPMI 8226 (panel B), MM.1 (panel C) and MM.1R (panel D) by 27%, 23%, 45% and 51% respectively. At 10  $\mu$ M curcumin completely suppressed the growth in all cell lines. These results indicate that curcumin suppresses the proliferation of all multiple myeloma cell lines tested, including MM.1R that is resistant to dexamethasone-induced apoptosis.

The antiproliferative effects of curcumin was also examined by thymidine incorporation in U266 cells. Curcumin suppressed thymidine incorporation within 24 h in a dose-dependent manner (Fig. 6A). Results in MTT assays, which indicates 5 mitochondrial activity of the cells, showed that curcumin suppressed mitochondrial activity of U266 cells within 24 h and the suppression occurred in a dose-dependent manner (Fig. 6B).

10

#### EXAMPLE 14

##### Curcumin Induces Apoptosis In Multiple Myeloma Cells

Whether suppression of NF- $\kappa$ B in multiple myeloma cells also leads to apoptosis was investigated. One of the hallmarks of 15 apoptosis is activation of caspases. U266 cells were treated with curcumin for different times, and whole cell extracts were prepared and analyzed for activation of caspase-9 (an upstream caspase), caspase-7 (a downstream caspase) and cleavage of PARP, a well-known substrate for caspase-3, -6 and -7.

20

Immunoblot analysis of extracts from cells treated with curcumin clearly showed a time-dependent activation of caspase-9

(Fig. 6C) and caspase-7 (Fig. 6D) as indicated by the disappearance of the 47-kDa and 35-kDa bands, respectively. Activation of downstream caspases lead to the cleavage of a 118-kDa PARP protein into an 89-kDa fragment, another hallmark of cells undergoing apoptosis (Figure 6E), whereas untreated cells did not show any PARP cleavage. Increasing amount of the 89-kDa fragment was also detected by antibodies that recognize only the cleaved 89-kDa PARP species (Fig. 6E, lower panel). Taken together, these results clearly demonstrate that curcumin induces apoptosis in multiple myeloma cells.

#### EXAMPLE 15

##### Curcumin Arrests Cells At The G1/S Phase Of The Cell Cycle

D-type cyclins are required for the progression of cells from the G1 phase of the cell cycle to S phase (DNA synthesis). Since a rapid decline of cyclin D1 was observed in curcumin-treated multiple myeloma cells, the effect of curcumin on U266 cell cycle was determined next.

Flow cytometric analysis of DNA from curcumin-treated cells showed a significant increase in the percentage of cells in the

G1 phase (from 52% to 70%) and a decrease in the percentage of cells in the S phase (from 22% to 9%) within 24 h of treatment with 10  $\mu$ M curcumin (Figure 7). These results clearly show that curcumin induces G1/S arrest of the cells.

5

#### EXAMPLE 16

NEMO-Binding Domain (NBD) Peptide Suppresses Constitutive NF- $\kappa$ B

10 And Proliferation Of Multiple Myeloma Cells

IKK is composed of IKK $\alpha$ , IKK $\beta$  and IKK $\gamma$  (also called NEMO). The amino-terminal  $\alpha$ -helical region of NEMO has been shown to interact with the C-terminal segment of IKK $\alpha$  and IKK $\beta$ . A small peptide from the C-terminus of IKK $\alpha$  and IKK $\beta$  NEMO has been shown to block this interaction. To make it cell permeable, the NBD peptide was conjugated to a small sequence from the antennapedia homeodomain. This peptide has been shown to specifically suppress NF- $\kappa$ B activation. The peptide without the antennapedia homeodomain protein sequence was used as a control.

15 Results disclosed above have shown that curcumin suppressed constitutive NF- $\kappa$ B which in turn led to suppression of

cell proliferation and induction of apoptosis. Here the NBD and control peptide was used to establish that NF- $\kappa$ B suppression is linked to proliferation and apoptosis.

As shown in Fig. 8A, treatment of U266 cells with NEMO-  
5 control peptide had no effect, whereas NBD peptide suppressed constitutive NF- $\kappa$ B in a time-dependent manner with complete suppression occurring at 12 h. Suppression of NF- $\kappa$ B activation in multiple myeloma cells was also confirmed independently by immunocytochemistry. The results indicated a decrease in the  
10 nuclear pool of the p65 subunit of NF- $\kappa$ B (Fig. 8B). Suppression of NF- $\kappa$ B by NBD peptide also led to inhibition of cell proliferation of U266 cells. Approximately 32% suppression of cell growth was observed after 24 h of NBD treatment (Fig. 8C). These results thus indicate that NF- $\kappa$ B suppression is indeed linked to antiproliferative  
15 effects in multiple myeloma cells.

#### EXAMPLE 17

#### Curcumin Potentiates The Cytotoxic Effects Of Vincristine

20 Because NF- $\kappa$ B has been implicated in chemoresistance of cells, the effects of curcumin on chemosensitivity was investigated.

Vincristine was chosen because it is one of the chemotherapeutic agents used for the treatment of multiple myeloma. Treatment of U266 cells with vincristine in the presence of low concentrations of curcumin (10  $\mu$ M) decreased cell viability after 24 h (Fig. 9). The 5 highest concentration (50  $\mu$ M) of vincristine alone was minimally effective in killing U266 cells; curcumin alone killed approximately 35% of the cells; whereas the two agents together killed 65% of the cells. These results indicate that curcumin may sensitize multiple myeloma cells to the cell killing effects of vincristine.

10

#### EXAMPLE 18

15 NF- $\kappa$ B Is Constitutively Active In CD138 $^{+}$  Cells From Multiple Myeloma Patients

This example examined whether NF- $\kappa$ B and STAT3 are constitutively active in fresh cells from multiple myeloma patients. Table 1 describes the clinical characterization of these patients. PBMC from normal subjects were used as a control.

20 CD138 $^{+}$  plasma cells from bone marrow of multiple myeloma patients were isolated as follows. CD138 antigen, also

known as Syndecan-1, is expressed on normal and malignant plasma cells, but not on circulating B cells, T cells, and monocytes. Anti-CD138 microbeads (Miltenyi Biotec, Auburen, CA) were used for positive selection of CD138<sup>+</sup> cells from bone marrow derived from 5 multiple myeloma patients. Two to 10 ml bone marrow sample was aspirated from the upper iliac crest or sternum and diluted in an equal volume of HEPES-buffered cell culture medium, IMDM, supplemented with heparin at a concentration of 100 U/ml, and mixed gently. To prevent the cells from clumping, the dilute 10 marrow was suspended in IMDM containing 100 U deoxyribonuclease (Dnase) I/ml and shaken gently at room temperature for an additional 30 min. Next, 30 ml of dilute bone marrow cell suspension was layered over 20 ml of Ficoll-Paque in 50 ml conical tubes and spun at 400x g for 30 min to isolate 15 mononuclear cells (MNC). Thereafter, the MNC layer at the interface were harvested and washed twice with PBS containing 2 mM EDTA for 10 min at 300x g at room temperature.

The MNC concentration was adjusted to 10<sup>7</sup> per 80  $\mu$ l of 20 Running buffer (PBS with 2 mM EDTA plus 0.5 mM BSA). For every 10<sup>7</sup> MNC in 80  $\mu$ l of running buffer, 20  $\mu$ l of anti-CD138 microbeads (Miltenyi Biotec, Auburen, CA) were added and the cell suspension

was incubated at 4 °C-8 °C for 15 min. The cell suspension was then diluted with 1 ml of cold Running buffer and centrifuged at 300x g for 10 min at 4 °C. The supernatant was discarded, and the cell pellet was suspended in 1 ml Running buffer, and loaded onto the 5 magnetic column of the AutoMACS system (Miltenyi Biotec) placed in a laminar flow hood.

Anti-CD138<sup>+</sup> cells were isolated by positive selection. The purity of the isolated CD138<sup>+</sup> plasma cell population was determined by treating 10<sup>5</sup> CD138<sup>+</sup> cells with 10 µl of anti-CD138 10 conjugated with phycoerythrin (PE) and incubated in the dark in the refrigerator at 6 °C -12 °C. The cells were washed twice with cold PBS, fixed with 1% paraformaldehyde, and analyzed using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA).

The NF-κB status of various multiple myeloma cell lines 15 was examined first. Figure 10A indicates all the multiple myeloma cell lines expressed the nuclear form of NF-κB, indicating that multiple myeloma cell lines express the constitutively active form of NF-κB. PBMC (the control) expressed the cytoplasmic (inactive) form of NF-κB (Figure 10B, upper panel). Multiple myeloma cells 20 from patient #1, like multiple myeloma cell lines, expressed only the nuclear form of NF-κB (Figure 10B, lower panel).

Twenty-two different multiple myeloma patient samples were then examined for NF- $\kappa$ B activation by the method described above. All 22 patients showed expression of NF- $\kappa$ B protein (p65) in the nucleus, indicating constitutive activation (Figure 11). The 5 extent of activation, however, was quite variable. Three patients showed low, five showed moderate and 14 showed high expression of constitutive NF- $\kappa$ B.

The constitutive activation of NF- $\kappa$ B was independently confirmed by the electrophoretic mobility shift assay. As shown in 10 Figure 11B, control NF- $\kappa$ B in KBM-5, a myeloid cell line which has no constitutive NF- $\kappa$ B, was activated by TNF. Similarly, NF- $\kappa$ B activation was also found in the sample from patient #4, which showed constitutive NF- $\kappa$ B activation by immunocytochemistry (Figure 11B).

15

20

**TABLE 1**  
**Clinical Characteristics of Multiple Myeloma Patients**

Patient #	Age, Sex	MM Type	Hgb	WBC	Platelets (1000)	% Plasma cells	Serum Paraproteins (gm)	Urine Paraproteins (gm)	Site
1	67 M	IgG	10.3	4.2	203	44	6.4	0.6	Bone (diffuse)
2	40 F	IgG	8.6	5.0	49	90	9.3	3.4	(-) bone survey
3	57 F	IgG	10.2	5.3	217	90	6.7	4.25	Bone (diffuse)
4	50 M	IgA	11.1	4.9	184	40	5.7	0	Bone (diffuse)
5	52 M	IgG	12.1	2.9	257	35	4.4	0.12	(-) bone survey
6	56 F	IgG	8	4.0	33	96	8.4	1.68	(-) bone survey
7	63 F	IgG	10	5.5	336	45	9.9	0.36	(-) bone survey
8	63 M	IgG	12.3	4.6	151	18	(-)	0.02	Bone (diffuse)
9	64 M	IgG	10.5	4.2	219	50	3.5	0.01	T11, Clavicle
10	35 F	IgA	9.1	2.6	85	7	1.5	0.21	(-) bone survey
11	52 M	IgA	10.4	3.3	54	66	0.8	17	(-) bone survey
12	45 M	IgG	10.2	11.8	338	63	0.1	4.273	Bone (diffuse)
13	54 M	IgG	14.1	4.5	262	14	4.7	0	(-) bone survey
14	66 M	IgA	10.2	5.9	154	60	3.4	0.1	Bone (diffuse)
15	50 F	IgG	11.9	7.1	364	18	3.7	0	Bone (diffuse)
16	40 F	IgG	10.3	6.2	335	60	(-)	0	Skull, Apex
17	58 M	IgA	13.9	5.1	205	20	4.3	0	(-) bone survey
18	67 M	IgG	8.9	8.8	52	25	0.4	3.9	(-) bone survey
19	56 M	IgG	13.3	7.8	233	22	4.6	0.02	Ribs
20	65 M	IgG	13.3	4.8	227	40	2.8	0.32	C2
21	57 M	IgA	6.0	9.6	219	68	3.1	4.8	(-) bone survey
22	67 F	IgG	12.8	5.0	249	14	1.6	0	(-) bone survey

MM, multiple myeloma; Hgb; hemoglobin; WBC, white blood cells.

## **EXAMPLE 19**

### **STAT3 Is Constitutively Active In CD138<sup>+</sup> Cells From Multiple**

#### **5 Myeloma Patients**

Only U266 cells expressed STAT3 in nuclei (Figure 12A), suggesting that U266 cells express the constitutively active form of STAT3. PBMC from normal subjects expressed the cytoplasmic (inactive) form of STAT3 (Figure 12B, upper panel). Multiple 10 myeloma cells from patient #1 likewise expressed the nuclear form of STAT3. This suggests that fresh cells from this patient express constitutively active form of STAT3 (Figure 12B, lower panel).

The status of STAT3 activation was examined in CD138<sup>+</sup> cells from 22 multiple myeloma patients. Unlike NF-κB, not all 15 patients showed expression of STAT3 protein in the nucleus (Figure 12C). The extent of nuclear STAT3 also varied. One patient had none, three had low, five had moderate, and 14 patients showed high expression of constitutive STAT3 activation.

## EXAMPLE 20

### Curcumin Downregulates Constitutive NF-κB And STAT3 Activation In CD138<sup>+</sup> Cells From Multiple Myeloma Patients:

5           Results presented above indicate that CD138<sup>+</sup> cells from most multiple myeloma patients expressed constitutively active NF-  
κB and STAT3. This example investigated whether curcumin suppressed the constitutive activation of NF-κB and STAT3 in fresh cells from multiple myeloma patients. To determine this, CD138<sup>+</sup>  
10    cells from multiple myeloma patients were exposed to 50 μM curcumin for 2 h and then examined for STAT3 and NF-κB expression.

Figure 13A indicates that NF-κB was constitutively active in patient # 5, #9 and #10 (the only patients tested) and exposure to curcumin downregulated NF-κB. Results in Figure 13B indicate that STAT3 was likewise constitutively active in patient # 5, #9 and #10, and exposure to curcumin downregulated this transcription factor.

## EXAMPLE 21

### Curcumin Downregulates The Survival of CD138<sup>+</sup> Cells From Multiple Myeloma Patients

5           Because NF- $\kappa$ B and STAT3 activation have been implicated in cell survival, and curcumin downregulated these transcription factors in CD138<sup>+</sup> cells from multiple myeloma patients, it is of interest to investigate whether this downregulation leads to a decrease in cell viability. Cells were exposed to different  
10      concentrations of curcumin and then examined for cell viability by the MTT method. As shown in Figure 14, curcumin treatment of U266 cells or fresh cells from patients #7, #9 and #10 decreased cell survival in a dose-dependent manner. Results in Figure 14B indicate that STAT3 was also constitutively active in patient # 5, #9  
15      and #10 and that exposure to curcumin downregulated this transcription factor. These results suggest that constitutive activation of NF- $\kappa$ B and STAT3 are cell survival factor for CD138<sup>+</sup> cells from multiple myeloma patients.

## EXAMPLE 22

### Dexamethasone Downregulates Constitutive NF-κB And STAT3 Activation In CD138<sup>+</sup> Cells From Multiple Myeloma Patients

5           Currently, dexamethasone is used as a standard therapy of multiple myeloma patients. Whether dexamethasone also affects NF-κB and STAT3 in cells from multiple myeloma patients was investigated. Results in Figure 15 indicate that dexamethasone downregulated the constitutive activation of both NF-κB (Figure 10 15A) and STAT3 (Figure 15B) in CD138<sup>+</sup> cells from patient # 20. Dexamethasone was, however, less effective than curcumin in downregulation of either transcription factor.

15           Dexamethasone also affects survival of cells from multiple myeloma patients. Results in Figure 16A indicate that dexamethasone decreased the survival of cells from patient # 20. Dexamethasone, however, was much less effective than curcumin (Figure 16B).

20           That dexamethasone can suppress NF-κB activation has been previously reported. The present study is the first to show the effect of dexamethasone on STAT3. Curcumin was much more effective in inhibiting the survival of multiple myeloma cells than

dexamethasone (Figure 16). Because of the established pharmacological safety of curcumin and its ability to downregulate expression of large number of genes involved in cell survival and chemoresistance, it provides sufficient rationale to combine 5 curcumin with dexamethasone for the treatment of multiple myeloma patients. Recently, a proteosomal inhibitor (PS341, called Velcade) and an inhibitor of TNF production (thalidomide) have been approved for the treatment of multiple myeloma patients. Both of these inhibitors have also been shown to suppress NF- $\kappa$ B 10 activation. The results presented herein suggest that NF- $\kappa$ B and STAT3 are ideal targets for drug development for the treatment of multiple myeloma.

The following references were cited herein:

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are 5 incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.